MOUSE MONOCLONAL ANTIBODY (5-21-3) TO HUMAN IMMUNODEFICIENCY VIRUS gp41 PROTEIN

BACKGROUND OF THE INVENTION

The present invention relates to the detection in body fluids such as blood or other blood products of exposure to the etiological agent of acquired immunodeficiency syndrome (AIDS). The etiological agent of AIDS is the human exogenous retrovirus termed Human Immunodeficiency Virus (HIV I), formerly named Human T-Cell Lymphotrophic Virus Type III (HTLV III), Lymphadenopathy Associated Virus (LAV), or AIDS Associated Retrovirus (ARV). In particular, a mouse monoclonal antibody, 5-21-3, and its native, conformation-dependent epitope within HIV I gp41 are provided which form the basis of an immunoassay used for the detection of exposure to HIV I. In another aspect, the invention relates to a monoclonal antibody for use as a probe against native antigens and synthetic peptides of HIV I.

Because AIDS is an infectious disease transmitted by parenteral exposure to contaminated blood or blood products (or intimate sexual contact), there is an established need to screen all blood and blood products prior to transfusion to detect and eliminate those derived from individuals exposed to HIV I. (See, for example Curran et al., Science (1985) 229:1352-1357; Council on Scientific Affairs, JAMA (1985) 254:1342-1345; Fauci et al., Ann. Int. Med. (1985) 102:800-813; Fauci et al., Int. Arch. Allergy Appl. Immun. (1985) 77:81-88. Diagnostic tests may determine if an individual is infected

with HIV I by in vitro recovery of infectious virus from the individual. In addition, diagnostic tests may detect HIV I proteins or nucleic acid in body fluid or tissue, indicating possible HIV I infection. However, most diagnostic tests which are presently used for mass screening of blood and blood products are designed to detect antibody to HIV I, thus establishing prior exposure to HIV I.

The most widely used antibody test is the enzyme-linked immunoabsorbent assay (ELISA) employing inactivated whole virus cultured in a cell line capable of virus replication as the antigen reagent. While this method is quite sensitive, and there is a high likelihood that a truly infected person will be detected, there is also the possibility that persons never exposed to the virus may occasionally give positive results.

The detection of false positives in screening assays of blood products is one of the most pressing problems with the commonly used methods. Presently, specimen samples are screened by an ELISA method and if the result is positive, two more tests are run on the sample. If either is positive, the specimen is said to be repeatedly reactive. The Western Blot is the method commonly used to determine whether repeatedly reactive specimens contain antibodies to HIV. However, low value repeatedly reactive specimens often do not give a positive result on the Western Blot (Dassey et al.,JAMA,255, 743-745 (1986)). Nevertheless, the blood product must be disposed of because doubt exists regarding its infectivity (Chalmers et al., JAMA,256, 1778-1783 (1986)). These false positives are often due to nonspecific binding of immunoglobulins to cellular protein in the viral isolates.

Safety is another concern associated with the ELISA assay method because the procedures entail culturing live virus in vitro and subsequent isolation and deactivation to provide whole virus reagent.The infectious nature of the HIV virus makes manufacturing the virus potentially hazardous.

Scientists are currently developing second-generation tests in an effort to overcome the aforementioned problems associated with

current assay methodologies. One approach has been the use of genetically engineered HIV antigens. Alternatively, tests may employ labeled antibodies directed toward the immunogenic epitopes of HIV I as competitive probes against antibodies in donated blood (or blood products). In addition, antibodies may be used as probes for detection of viral antigens. By identifying immunogenic HIV I proteins and their native, immunodominant regions or epitopes, and raising monoclonal antibodies specific to these epitopes, highly sensitive and specific diagnostic tests to determine exposure to HIV I can be produced. Additionally, the use of monoclonal antibodies as test reagents in diagnostic assays for AIDS avoids the hazard of working with the whole virus.

SUMMARY OF THE INVENTION

It has now been found that mouse monoclonal antibody 5-21-3, and its native conformation-dependent, non-linear epitope within HIV I gp41 form the basis of an immunoassay used for the detection of exposure to HIV I in body fluids. The amino acids of HIV I gp41 which form the contact residues with the antigen combining site of the monoclonal antibody are located between residues 131 and 154 (amino acids 649 thru 672 as numbered by Ratner et al., Nature (1985) 313:277-283). The monoclonal antibody identifies a native antigenic determinant which cannot be mimicked by a linear peptide comprising the region (131-154), but can be partially mimicked by peptides comprising this region which also contain flanking sequence of varying length at the 3' and/or 5' ends. The additional flanking sequence either 5' (amino end) of amino acid 131 or 3'(carboxy end) of amino acid 154, is required to put the linear amino acid sequence (131-154) in the proper antigenic conformation.

Appropriate labeling (for example, isotope, enzyme or fluorescein) of the monoclonal antibody yields a highly specific reagent which can be employed in immunoassays designed to detect prior

exposure to HIV. In presently preferred forms, serum, plasma, saliva, or other biological samples collected from the patient are analyzed by immunoassay techniques such as radioimmunoassays,fluorescent immunoassays, or enzyme-linked immunosorbent assays in either direct or competitive forms. In an especially preferred assay procedure of the invention the design of the immunoassay places the labeled monoclonal antibody in competition with antibody positive sera (containing anti-HIV I gp41 antibody) for binding to the native epitope recognized by the monoclonal antibody. The monoclonal antibody can also be used as a probe against native antigens and synthetic peptides of HIV. Monoclonal antibody mapping to this general region (131-154), has not been previously identified as clinically useful for detection or evaluation of prior exposure to HIV I.

DESCRIPTION OF THE DRAWINGS

Fig. 1 is an autoradiograph showing the specificity of monoclonal antibody 5-21-3 for HIV I gp160/41 by immunoprecipitation of metabolically radiolabeled gp160.

Fig. 2 is a Western Blot showing specificity of monoclonal antibody 5-21-3 for HIV I gp41.

Fig. 3 is a photograph of microscope slides showing specificity of monoclonal antibody 5-21-3 for HIV I by immunocytochemical analysis.

Fig. 4 is an illustration of the location of the constructed synthetic peptides (P) on the HIV I ENV gene, which were employed to map and characterize the epitope recognized by monoclonal antibody 5-21-3.

Fig. 5 is an immuno-dot blot analysis of the mapping of the epitope recognized by monoclonal antibody 5-21-3.

Fig. 6 illustrates the epitope mapping of monoclonal antibody 5-21-3 using synthetic peptides in a binding inhibition assay. Selected concentrations of synthetic peptides P58-130 (▲), P67-154 (●), and P121- 154 (■) were tested for their relative ability to block binding of monoclonal antibody 5-21-3 to recombinant-derived p41.

DETAILED DESCRIPTION

The present invention provides novel means for determining the presence of HIV infection in body fluids through the use of a monoclonal antibody characterized by its specificity for an epitope on HIV I gp41 formed by a first sequence of amino acids Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys with at least one flanking amino acid sequence of at least 5 amino acids in length either 3'to the carboxy terminus or 5' to the amino terminus of the first sequence, with the flanking sequence having an amino acid sequence substantially corresponding to that found on native HIV I gp41 adjacent said first sequence. In a preferred embodiment of the present invention, the monoclonal antibody ("5-21-3") is described, which is useful as a test reagent in diagnostic assays. In presently preferred forms, body fluid samples from patients are analyzed by immunoassay techniques such as radioimmunoassays, fluorescent immunoassays, or enzyme-linked immunosorbent assays in either direct or competitive formats.

In another of its aspects, the present invention provides novel hybridoma cell lines (exemplified by murine-derived cell line ATCC HB -9628) and novel monoclonal antibodies secreted thereby (exemplified by the above-noted 5-21-3 monoclonal antibody). Antibodies of the invention may be unlabeled or labeled with suitable detectable immunoassay markers such as radiochemicals, enzymes, fluorescent compounds or chemiluminescent compounds.

In an especially preferred embodiment of the invention labelled 5-21-3 monoclonal antibody is employed in an

enzyme-immunoassay (EIA) as a competitive probe against anti-HIV I antibody in serum samples for binding to full length recombinant derived HIV I gp41 bound to a solid support. The labeled 5-21-3 monoclonal antibody can also be employed in the Abbott ENVACOR Assay as disclosed in U.S.Patent Serial No. 020,282.

In addition to the general competitive binding assay techniques described in detail herein, immunoassays within the scope of the present invention include any assay format which is dependent on the formation of an antigen/antibody complex. Such assay formats include, for example, agglutination assays and solid phase immunometric assays such as the forward sandwich, reverse sandwich and simultaneous sandwich assays. Any of the above assay formats can be utilized for the detection of antibodies to HIV I as well as the detection of the HIV I virus or portions thereof.

Biological samples which are easily tested by the methods of the present invention include human and animal body fluids such as whole blood, serum, plasma, urine, saliva, lymphocyte or cell culture preparations, and purified and partially purified immunoglobulins. Solid supports which can be used in the immunoassays of the invention include wells of reaction trays, test tubes, beads, strips, membranes, microparticles or other solid supports which are well known to those skilled in the art. Any label capable of producing a detectable signal can be used in the assays of the invention. Representative labels include enzymatic, radioisotopic, fluorescent and chemiluminescent labels. Further, hapten/labeled anti-hapten systems such as a biotin/labeled anti-biotin system may be utilized in the inventive assays. Alternatively, one may employ a labeled specific binding partner for the antibody which may be, for example, a labeled antibody specific for the antibody employed or labeled antigen that binds to the antibody. Where the monoclonal antibody is derived from a murine source, a labeled anti-mouse immunoglobulin specific for the antibody employed in the method may be used. Additionally, the antigen reagent may be composed of inactivated whole virus, or partially purified, native, synthetic, or recombinantly-produced HIV I protein.

In addition, the materials for use in the assays of the invention are ideally suited for preparation of a kit. Such a kit may comprise a carrier means being compartmentalized to receive in close confinement one or more container means such as vials, test tubes, and the like. Each of the container means comprises one of the separate elements to be used in the method.

The following illustrative examples relate to the development of monoclonal antibodies which specifically react with an antigenic determinant of gp41 of HIV I. More specifically, Examples 1 and 2 relate to procedures whereby hybridoma cell lines secreting monoclonal antibodies were generated. Example 3 relates to characterization of monoclonal antibody 5-21-3 produced by a selected hybridoma cell line. Example 4 relates to an amplification method for producing greater amounts of the 5-21-3 antibody. Example 5 relates to assays performed to determine the activity and specificity of the 5-21-3 monoclonal antibody. Example 6 relates to an enzyme-linked immunoassay (EIA) based on the 5-21-3 monoclonal antibody and use of this EIA in detection of HIV seropositive individuals. Example 7 relates to the mapping and characterization of the gp41 epitope recognized by monoclonal antibody 5-21-3 . Cell line 5-21-3 was deposited at the American Type Culture Collection, Rockville, Maryland, on January 21, 1988, under deposit accession number HB-9628.

Example 1
Immunization of Mice

In the procedure for production of hybridoma cell line 5-21-3, BZH mice (obtained from Chella David, Mayo Clinic, Department of Immunology) were immunized with partially purified, detergent disrupted HIV I (HTLV III prototype strain obtained from R.C. Gallo, National Institutes of Health). HIV I was partially purified from infected H9 cells by (a) membrane filtration separating HIV I from cells, followed

by (b) concentration of cell culture fluid containing HIV I, followed by (c) collection of virus by ultracentrifugation, followed by (d) resuspension of the virus and collection by centrifugation onto a 20% sucrose pad, followed by (e) sucrose density gradient banding of HIV I at a density of approximately 1.16, and (f) ultracentrifugation of banded virus to collect and concentrate HIV I. HIV I was disrupted by addition of 0.5% Triton X-100, followed by vigorous sonication at 4°C.

On day 1, mice received 40 ug of disrupted HIV I with 10 ug of S.typhimurium extract (RIBI Immunochem. Research Inc.) in 0.2 ml of Freunds Complete Adjuvant (Difco Laboratories) given subcutaneously (s.c.) and intraperitoneally (i.p.). The second immunization was performed 18 days later when mice received 50 ug of HIV I and 10 ug of S.typhimurium extract given s.c. and i.p. in Freunds Incomplete Adjuvant (Difco Laboratories). For the 3rd, 4th, and 5th immunizations, at 34, 47, and 68 days after the first immunization, respectively, mice were immunized i.p. and s.c. with approximately 50 ug of HIV I in RIBI Adjuvant System (RIBI Immunochem. Research Inc.) containing 6,6'-dimycolyltrehalose and monophosphoryl lipid A. Mice were bled on days 26, 42, and 56. The immune response of immunized mice was assessed by assaying their sera for anti-HIV I antibody by enzyme-linked immunoassay and western blot.

A   Enzyme-linked Immunoassay (EIA)

Sera from naive or immunized mice were serially diluted into 20 mM potassium phosphate buffer, pH 7.4, containing 0.15 M NaCl, 5 mM EDTA, 20% normal goat serum, 10% fetal calf serum, 10 mM EGTA, and 0.2% Tween 20. The diluted sera were reacted against polystyrene beads directly coated with partially purified HIV I, or, alternatively, with HIV I bound to the bead via human anti-HIV I IgG (purified from serum of an HIV I seropositive individual). After 2 hours at 40°C, the beads were washed, and goat anti-mouse IgG (H+L) Horseradish Peroxidase (HRPO)- conjugated antibody was added. The beads were incubated 2

hours at 40°C, washed, and transferred to reaction tubes for addition of o-phenylenediamine:2HCl (OPD) color reagent. The reaction was stopped after 30 min. by addition of 1N H_2SO_4, and the absorbance at 492 nm was determined. The absorbance was directly proportional to the amount of mouse antibody bound to the beads. Reactivity of mouse sera to HIV I antigens on either bead was confirmed by addition of a human HIV I seropositive serum (20-50 ul) as a competitor for binding to HIV I. These assays demonstrated the presence of antibody to HIV I in the sera of immunized mice.

B. Western Blot

Approximately 500 ug of partially purified HIV I were treated with sodium dodecyl sulfate (SDS) and 2-mercaptoethanol at 95°C, and electrophoresed in a 12% polyacrylamide-SDS gel (Laemmli et al., Nature (1970) 227:680-685). Proteins were transferred overnight from the gel to nitrocellulose by electrophoresis at 100 mamp, or transferred in 1-2 hr at 1.0 amp, in standard transfer buffer composed of 25 mM Tris (Hydroxymethyl) Aminomethane, 192 mM glycine, and 2.0% methanol, pH 8.3. (Towbin et al., Proc. Natl. Acad. Sci. (1979) 76:4350-4354.) After transferring the viral proteins and blocking the nitrocellulose with 20% fetal calf serum diluted in 10 mM Tris buffer (pH 8.0) containing 0.15 M NaCl, the nitrocellulose was cut into strips (each strip containing approximately 15 ug of viral protein) which were used to determine the presence of anti-HIV I antibody in test sera (or other samples). Reaction mixtures consisted of a nitrocellulose strip incubated with an appropriate amount of test sample in 2.5 ml of buffer (20 mM Tris, 1 mM EDTA, 0.2 M NaCl, 0.3% Triton X-100, and 2 mg/ml bovine serum albumin, pH 7.5) for 1-2 hours at room temperature. The strips were washed with buffered detergent (10 mM phosphate buffered saline (PBS), pH 7.5, containing 0.1% SDS and 0.5% Triton X-100, alternated with PBS containing 0.5 M NaCl and 0.5% Triton X-100), followed by addition of goat anti-mouse IgG antibody conjugated to HRPO. The strips were incubated for 1-2 hours at room temperature,

followed by washing with buffered detergent. Finally, antibody bound
to viral protein was visualized by addition of freshly prepared HRP
color reagent (Biorad) (120 mg dissolved in 40 ml ice-cold methanol,
then diluted into 200 ml Tris buffered saline, pH 7.8, containing 120
ul of 30% hydrogen peroxide). This assay demonstrated the presence of
antibody to specific HIV I proteins.

Example 2
Cell Fusion

Upon demonstration of specific anti-HIV I antibody present at
reasonable titers in sera of immunized mice, the mice were allowed to
rest for at least 3 weeks prior to a pre-fusion boost of antigen. The
pre-fusion antigen boost was performed by intravenous (tail vein)
injection of approximately 40 ug of disrupted HIV I. Three days later
the mice were sacrificed, and their spleens, containing anti-HIV I
antibody producing cells, were disrupted to single cells. The single
cell suspensions were treated with 0.83% NH_4Cl to remove red blood
cells, and then mixed with SP2/0 cells at a 10:1 (SP2/0:spleen cells)
ratio. The mixed cells were centrifuged, washed once with serum-free
medium, then centrifuged again. The fusogen, polyethylene glycol
(PEG), was used to form hybrids of the immune donor spleen cells with
the myeloma cell line SP2/0 (HPRT neg.) [G. Kohler and C. Milstein,
Nature (1975) 256:494, and reviewed in Monoclonal Hybridoma Antibodies:
Techniques and Applications, ed. J.G.R. Hurrell (CRC Press, Inc.,
1982)]. Briefly, fusion of the spleen and SP2/0 cells was accomplished
by exposing the pellet to 40% PEG (ATCC, MW 1300-1600) in serum-free
Iscoe's Modified Dulbecco's Medium (IMDM) for two minutes. The PEG and
cell suspension was diluted slowly by the addition of 20 ml of serum-
free IMDM over a period of five minutes, followed by collection of the
cells by centrifugation. The supernatant was decanted and replaced
with 30 ml IMDM containing 20% fetal bovine serum (Hyclone) with HAT

(hypozanthine, aminopterin, and thymidine) to select for hybridomas. Spleen cells from one nonimmune Balb/c mouse also were added as a feeder layer. The cells were plated at 0.1 ml/well in three 96 well tissue culture plates. Three days later an additional 0.1 ml of HAT media was added to each well. At weekly intervals thereafter, one-half the media was replaced with IMDM containing 20% fetal bovine serum (Hyclone) with HT (hypozanthine and thymidine), and hybrids were allowed to grow for an additional 7-14 days.

Some of the hybrids were composed of spleen cells making antibody to HIV I fused with SP2/0 cells. Briefly, the fusogen promotes fusion of spleen cell and SP2/0 cell membranes, forming a heterokaryon containing nuclei of both cells. Eventually, the dissimilar nuclei fuse producing a single nucleus capable of synchronous mitosis. As the fused cells divide, the hybrid stabilizes by losing chromosomes of each nuclei. The fused cells are plated into multiple 96 well plates at 10^5 to 10^6 cells per well. Hybrid cells formed from SP2/0:spleen cell fusions are selectively propagated by culturing in HAT medium. All unfused SP2/0 or SP2/0:SP2/0 fused cells are prevented from growing by aminopterin, and unfused spleen cells or spleen:spleen fused cells die off in culture. Only SP2/0:spleen hybrids will grow in the HAT selection medium.

Example 3
Screening, Cloning, and Characterization of Monoclonal Antibody

After 10-14 days, culture fluids from the wells containing cell growth (hybridomas) were screened for antibody to HIV I using procedures described in Example 1 (EIA and Western Blot). In addition, an EIA using gp41 produced in E .coli by recombinant DNA methods (rp41) was employed for screening. Briefly, polystyrene beads coated with human IgG (purified from an anti-gp41 seropositive individual) were

reacted with crude extracts of E. coli, previously induced for production of the HIV I gp41 cloned gene (BglII to KpnI restriction fragment, containing all the amino acids of gp41) in a lac z expression vector (Wood et al., Cold Spring Harbor Symposium (1985), p. 72: Wood et al., J. Cell. Biochem. Suppl. (1985) 9A:24). The resultant beads, bearing rp41 bound via human IgG, were reacted with hybridoma culture fluid for 2 hr at 40°C, washed, and then incubated with HRPO-labeled goat anti-mouse IgG antibody for 1 hr at 40°C. After washing again, beads were transferred to reaction tubes for addition of OPD substrate as described in Example 1.

From a single fusion (fusion #5), 16 wells were identified which contained cell growth and colony formation. Culture fluid from each well was screened by EIA and Western Blot. Of the 16 wells 7 were identified as possibly containing antibody to HIV I. Cells from each of these 7 were expanded in multiple 24 well plates, and culture media was again assayed for presence of anti-HIV I antibody. All 7 were positive for anti-HIV I antibody, and each hybrid was further expanded in T25 flasks for cloning by limiting dilution. Each expanded hybrid was plated into 96 well microtiter plates at dilutions of 10^5 to 10^6 and allowed to grow for 10-21 days. Among the clones identified as producing anti-gp41 antibody was clone #3 from hybrid #21 of fusion #5, which was named accordingly, 5-21-3. The clone was obtained by limiting dilution using the guidelines outlined by J.W. Goding in Monoclonal Antibodies: Principles and Practice (Academic Press, N.Y., 1983). The isotype of monoclonal antibody 5-21-3 was determined to be IgG1. The EIA isotyping procedure employed a microtiter plate coated with goat anti-mouse IgG immunoglobulin, which was incubated with culture fluid of the clone to capture the secreted mouse antibody. After 2 hours, the plate was washed, then rabbit anti-mouse isotype was applied for an additional 2 hr. The plate was washed again, and HRPO-conjugated goat anti-rabbit IgG was applied for 1 hr. The excess conjugate was removed by washing, then OPD substrate was added. The amount of rabbit anti-mouse isotype bound to the mouse immunoglobulin

was proportional to the absorbance measured at 492 nm. Further
characterization was performed with 5-21-3 antibody from mouse ascites.

Example 4
Amplification of Antibody Yields by Ascites Method

In order to obtain greater amounts of 5-21-3 monoclonal
antibody, ten to twenty million cloned 5-21-3 cells were innoculated
into a Balb/c mouse previously treated intraperitoneally with 0.5 ml
pristane (2,6,10,14-tetramethylpentadecane)[method outlined in Hurrell
et al., supra]. Pristane treatment enhances growth of mouse myeloma
hybrids within the peritoneum of the mouse, and the ascites fluids
which form are rich in the monoclonal antibody secreted by the hybrid
cells. After formation of monoclonal antibody-enriched ascites, the
mice were sacrificed, and the ascites withdrawn from the peritoneum was
clarified by centrifugation and stored at -20°C. The monoclonal
antibody in the ascites was titered by EIA (see Example 5). Other
characterization procedures (described herein) were performed with the
clarified ascites fluid or purified antibodies from the ascites, using
several purification procedures known in the art, including Protein
A-Sepharose (Hurrell et al., supra).

Example 5
Activity and Specificity

Assays were performed to determine the titer of monoclonal
antibody in mouse ascites and to assess the specificity of the
monoclonal antibody.

A. EIA

Assays described in Examples 1 and 3 were used to determine
the titer of monoclonal antibody in ascites fluid. Briefly, clarified
ascites fluid was reacted against polystyrene beads coated directly

with partially purified HIV I, or, against beads bearing HIV I proteins bound via human IgG . Using these assays, the titers of antibody in ascites fluid were relatively low (1:8000 to 1:16,000). However, the activity was determined to be against HIV I protein because serum from a seropositive AIDS patient was a strong competitive inhibitor of the monoclonal antibody (Table 1).

In addition, a second assay was employed which utilized rp41 produced in $\underline{E}$. $\underline{coli}$ by recombinant DNA methods (described in Example 3), and IgG from a goat immunized with HIV I. Goat anti-HIV I IgG bound to polystyrene beads was used to capture the rp41 (see Table 1, footnote d). The monoclonal antibody-enriched ascites exhibited high titers (>1:128,000) against the rp41 antigen. These results indicated that the monoclonal antibody was directed against an epitope present on HIV I gp41.

Table 1. Activity of Monoclonal Antibody 5-21-3 Enriched Mouse Ascites Against Disrupted HIV I and rp41.

Ascites Dilution	ABSORBANCE(492nm)			
	HIV coated bead[a]	AB+HIB coated bead[b]	Competition w/AIDS serum[c]	AB+rp41 bead[d]
1:2,000	0.214	0.291	0.114	1.149
1:4,000	0.222	0.247	0.074	1.042
1:8,000	0.171	0.165	0.059	1.062
1:16,000	0.130	0.160	0.039	0.825
1:32,000	0.099	0.099	0.032	0.687
1:64,000	0.054	0.067	ND[e]	0.678
1:128,000	0.023	0.062	ND[e]	0.434

[a]Partially purified, detergent disrupted HIV I was coated directly onto a polystyrene bead. The endpoint titer of 5-21-3 was considered to be 1:8,000 to 1:16,000.

[b]Anti-HIV I IgG purified from the serum of an AIDS patient was coated onto a polystyrene bead and used to capture HIV I proteins, which in turn served as the antigenic targets in the assay. The endpoint titer of 5-21-3 against these beads was considered to be 1:8,000 to 1:16,000.

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cSerum from an AIDS patient (anti-HIV I) was used to block reactivity of 5-21-3 ascites against the AB + HIV bead, thus confirming the monoclonal antibody was seeing the same antigens as HIV I immune human serum.

dIgG purified from the serum of a goat immunized with HIV I, was coated onto a polystyrene bead and used to capture rp41 produced in E. coli by recombinant DNA methods. The endpoint titer of 5-21-3 enriched ascites was considered to be greater than 1:128,000 against the rp41 coated beads.

eND, not determined.

B. Radioimmunoprecipitation (RIP)

Specificity of 5-21-3 antibody was confirmed by immunoprecipitation of $[^{35}S]$methionine/$[^{35}S]$cysteine biosynthetically-labeled viral glycoprotein gp160 (envelope precursor) from HIV I infected H9 cell lysates. Immunoprecipitation assays for viral proteins have been described previously (Devare et al., Proc. Natl. Acad. Sci., U.S.A. (1986) 83: 5718-5722). Cell lines employed for these studies were uninfected H9 cells, HIV I infected H9 cells, or a cloned cell line, S4C8, which was derived from HIV I infected H9 cells, but which produces primarily gp160, gp120, and gp41, and little or no gag gene products. Cells were harvested from culture, washed once with RPMI 1640 deficient in methionine and cysteine (Gibco Laboratories), then suspended at 1-2.5 x 10^6 cells/ml in the same medium. Washed cells were incubated for 30- 45 min. at 37°C in 6% CO_2, followed by addition of 50-100 uCi each of $[^{35}S]$methionine and $[^{35}S]$cysteine (Amersham) to the medium. Cells were radiolabeled at 37°C for 4-8 hr, harvested by centrifugation, and lysed in PBS, pH 7.4, containing 1 mM PMSF, aprotinin (100 Kallikrein inactivation units per ml of buffer), 1.0% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate (all reagents from Sigma). The lysate was clarified by centrifugation at 100,000 x g for 40 min. and stored at - 70°C.

Immunoprecipitation was performed by incubating 100 ul aliquots of cell lysates with 5-20 ug of purified monoclonal antibody (1mg/ml), 3-5 ul of monoclonal antibody-enriched ascites, 3-10 ul of

serum, or 100 ul tissue culture fluid, for 30-60 min. at 4°C.
Antibody-antigen complexes were recovered by addition of 200 ul of
pre-swollen Protein A-Sepharose (Pharmacia) previously washed in lysis
buffer containing 1mg/ml bovine serum albumin (IgG binding capacity of
50-200 ug/200 ul Protein A). The reaction mixture was shaken
vigorously at 4°C for 1 hr, followed by 3 washes of the Protein
A-Sepharose using lysis buffer. Protein A- Sepharose was then
collected by centrifugation, and immune complexes were dissociated by
heating at 95°C in SDS gel sample buffer containing 2-mercaptoethanol
(Laemmli et al., supra). The sample was subjected to SDS-10%
polyacrylamide gel electrophoresis. The gel was incubated for 30 min.
in Enhance (Dupont), dried, and exposed to x-ray film for
autoradiography of the immunoprecipitated, radiolabeled proteins. The
results are illustrated in Figure 1 where, in Panel A, cell lysates
from labeled HIV I infected H9 cells were mixed with 3 ul of 5-21-3
enriched ascites (A), 3 ul of a different (not 5-21-3) anti-gp160/41
monoclonal antibody enriched ascites (B), 3 ul of normal mouse serum
(C), 3 ul of a miscellaneous mouse ascites (D), or 3 ul of a HIV I
immune human serum (E). Molecular weight standards are in (F). In
Panel B, cell lysates from labeled S4C8 cells (C,E) or uninfected H9
cells (B,D) were mixed with 100 ul of tissue culture fluid containing
5-21-3 (B,C) or 5 ul of HIV I immune human serum (D,E). Molecular
weight standards are in (A). The results indicated that only gp160,
and not gp120, was precipitated from HIV I infected cell lysates by the
monoclonal antibody, and that there was no detectable cross reaction
with any other radiolabeled viral or cellular protein. Thus, the
monoclonal antibody 5-21-3 specifically bound HIV I gp160/41.

C. Western Blot

        Western blot analysis (as described in Example 1) of purified
monoclonal antibody 5-21-3 was performed to further establish
specificity. The results are illustrated in Figure 2 where
approximately 48 ug of partially purified HIV I was electrophoretically

transferred onto each nitrocellulose strip after electrophoresis in a 12% polyacrylamide gel under reducing (A,C,E,G,I) or non-reducing (B,D,F,H,J) conditions. The strips were incubated with 1.0% normal human plasma (A,B), 1.0% HIV I immune human serum (C,D), 15 ug/ml purified anti-HIV I gp41 monoclonal antibody 10-15-63 (E,F), 15 ug/ml monoclonal antibody 5-21-3 (G,H),or 15 ug/ml normal mouse IgG (I,J). The results indicated that 5-21-3 was specific for a single diffuse band of M_r 41K when reacted against nitrocellulose strips bearing HIV I proteins. No other non-specific reactions were demonstrated. These data were consistent with the previously demonstrated specificity of the antibody toward gp160/41.

D. Immunocytochemical Assay (ICA)

Monoclonal antibody 5-21-3 was employed in an immunocytochemical assay against uninfected and HIV I infected H9 cells in order to further demonstrate its specificity for HIV I. Briefly, uninfected or HIV I infected H9 cells were fixed to microscope slides using 100% acetone for 10 min., then air dried, and stored at -20°C. For staining, the cells were rehydrated in PBS for 10 min., followed by addition of 100 ul of 2% normal goat serum in PBS containing 100 ug/ml normal human IgG for 10 min. Excess reagent was removed, and 100 ul of 1 ug/ml 5-21-3 IgG was added for 30 min. to the slides maintained in a humidified chamber. The slides were washed in PBS, followed by addition of 100 ul of the second antibody, goat anti mouse IgG (Pel Freez) at a 1:400 dilution, for 30 min. The slides were washed with PBS, followed by addition of 100 ul of peroxidase anti-peroxidase complex (Sternberger and Meyer, Clonal PAP) diluted 1:300 in PBS containing 2% normal goat serum and 100 ug/ml normal human IgG. The slides were incubated (with the Clonal PAP) in a humidified chamber. After washing in PBS, color development was initiated by the addition of 100 ul of the chromagen (0.63 mg/ml tetra hydrochloride diamino-benzidine in 0.06% hydrogen peroxide in PBS). Color development was stopped after 6 min. by washing the slides in tap water. The slides were dehydrated and mounted for examination.

Results of the immunocytochemical analysis are illustrated in Figure 3 where the monoclonal antibody 5-21-3 (5 ug/ml) was reacted against (A) HIV I infected H9 cells (400x) and (B) uninfected H9 cells (400x). Normal mouse IgG (5 ug/ml) also was reacted against (C) HIV I infected H9 cells (250x) and (D) uninfected H9 cells (250x). Results indicated that 5-21-3 did not cross react with any normal cellular constituent. Specific staining was not detected when normal, uninfected H9 cells were employed as the target for 5-21-3 (panel B). In sharp contrast, a strong, characteristic pattern of staining was apparent when HIV I infected H9 cells were employed as the target (panel A). A characteristic "prominant body" was observed in the HIV I infected cells when the primary antibody was 5-21-3. This staining pattern was absent when normal mouse IgG was substituted for 5-21-3 IgG (panel C). In addition, the "prominant body" was absent when anti-HIV I p24 or anti-HIV I p17 monoclonal antibodies were used as the primary antibody (not shown), although they also exhibited characteristic staining patterns only against infected cells.

Example 6
Clinical Utility of a Competitive EIA Employing Monoclonal Antibody 5-21-3 for Identification of HIV I Seropositive Individuals

A. Competitive EIA

Monoclonal antibody 5-21-3 was purified from mouse ascites using Protein A-Sepharose, and coupled to horseradish peroxidase (HRPO). Briefly, the HRPO was activated by addition of 1.6 ml of 5 mg/ml HRPO (Sigma) to 1.6 ml of $NaIO_4$ at 17 mg/ml. The mixture was incubated 30 min. at 30°C in the dark, followed by separation of activated HRPO from $NaIO_4$ by G25 Sephadex gel filtration. Fractions containing activated HRPO were pooled and protein concentration was adjusted to approximately 0.6 mg/ml. One ml of 1 mg/ml purified 5-21-3 IgG, previously dialyzed into 50 mM sodium carbonate buffer, pH 9.5,

was added to 1.1 ml of activated HRPO at 30°C and incubated 2 hr in the dark, followed by a 30 min. incubation at 4°C. The theoretical coupling ratio of HRPO:IgG in the reaction mixture was 4:1. Freshly prepared Na_2BH_4 (0.08 ml of 5 mg/ml solution in cold 10 mM Na_2CO_3, pH 9.5) was added to the mixture and left overnight at 4°C, followed by addition of 0.08 ml acetone which was allowed to react for 30 min. at 30°C. Finally, 7.7 ml of 1.43% bovine serum albumin in PBS (pH 7.2) were added to yield a final IgG concentration of 100 ug/ml; aliquots were stored at -70°C.

The conjugated monoclonal antibody ([HRPO]5-21-3) was employed in an EIA as a competitive probe (Monoclonal Assay) against anti-HIV I antibody in serum samples, for binding to full-length recombinant-derived HIV I rp41. Polystyrene beads were coated with human IgG (purified from sera of an HIV I seropositive individual having strong anti-gp41 activity), and then incubated with clarified E. coli extracts, previously induced for production of full-length rp41 (as described in Example 3). The beads bearing rp41 were used as serological targets for competition between [HRPO]5-21-3 and anti-HIV I seropositive samples. Beads were added to the wells of reaction trays (Abbott Laboratories) containing 50 ul of the test sera and 200 ul of [HRPO]5-21-3 diluted in PBS (pH 7.4) containing 10% normal human plasma, 10% fetal calf serum, 20% normal goat serum, 10% Triton X-100, and 4.77 g/L of HEPES buffer. This reaction mixture was incubated for 4 hr at 40°C, followed by vigorous washing, transfer of the bead to a reaction tube, and addition of 0.3 ml of OPD as substrate for color development. After 30 min., color development was stopped by addition of 1 ml of 1N H_2SO_4, and the absorbance value at 492 nm was determined. The absorbance value was directly proportional to the amount of [HRPO]5-21-3 bound to the bead.

In the presence of competing anti-HIV I gp41 antibody in the serum sample, the amount of [HRPO]5-21-3 bound to the bead was significantly decreased. A cutoff value (c/o) was established as c/o = 0.5(NC-PC), where PC (positive control) is the assay absorbance value

for an established anti-gp41 seropositive human serum, and NC (negative control) is the absorbance value for an established anti-gp41 seronegative serum. Test sera with values less than the c/o value were considered positive for anti-gp41 antibody because they blocked binding of [HRPO]5-21-3 to the target rp41, and sera with values above the c/o value were considered seronegative for anti-gp41 antibody. In addition, in order to account for daily fluctuations in the c/o value, the clinical data were expressed as sample:c/o ratios, where the assay A_{492} value of the sample was divided by the daily assay c/o value. Seropositive samples exhibited ratios of <1.0, whereas seronegative samples exhibited ratios of >1.0.

B. Clinical Utility

The clinical utility of the Monoclonal Assay was assessed by comparing it to a similar assay (Abbott ENVACOR Assay) of established clinical efficacy (Dawson et al., J. Infect. Dis. (Jan.,1988) 157:149-155 ; Frosner et al., Lancet (1987) i:159-160; Goudsmit et al., J. Infect. Dis. (1987) 155:558-560; and Habermehl et al., Infection (1987) 14:216). The configuration of the envelope (ENV) portion of the ENVACOR assay was nearly identical to the Monoclonal Assay except that the envelope assay employed human, polyclonal anti-HIV I IgG (purified from serum of an HIV I gp41 seropositive individual) as the HRPO-conjugated competitive probe (ENV Polyclonal Assay). Table 2 presents the results of several population studies comparing the Monoclonal and ENV Polyclonal Assays. The Monoclonal Assay was highly effective in detecting points of seroconversion in longitudinal studies, and the utility of the Monoclonal Assay was not affected by the geographic source of the specimen, nor by the disease state of the individual. In addition, the Monoclonal Assay was less susceptible to false negative results than the ENV Polyclonal Assay in the study (see footnote f).

Table 2. Population Studies Comparing the Clinical Efficacy of
Polyclonal (Abbott ENV Assay) vs. Monoclonal (Mab 5-21-3) Competitive
Assays for Detection of Anti-HIV I gp41 Antibody.

Source of Sera	No. of Individuals	No. of Serum Samples	Donor Status	ENV+ Mab+	ENV- Mab-	ENV+ Mab-	ENV- Mab+
Chicago USA	120	152	AIDS-28	38	0	0	0
			ARC-10	16	0	0	0
			ASYM-80	56	39	[a]1	0
			Other-2	[b]1	[c]1	0	0
California USA	Unknown[d]	19	AIDS-10	9	1	0	0
			LAS-10	10	0	0	0
Berlin W. Germany[e]	31	122	AIDS-6	22	5	0	0
			ARC-8	30	5	0	[f]2
			ASYM-9	20	11	0	0
			Other-8	22	4	0	[g]1
W. Germany	Unknown[d]	59	AIDS-10	10	0	0	0
			ARC-10	10	0	0	0
			ASYM-30	30	0	0	0
			Other-9	9	0	0	0
Rome, Italy	35	35	Unknown	15	20	0	0
Zaire, Africa	40	40	Varied	[h]2	[h]38	0	0
Amsterdam, Netherlands[j]	32	202	High Risk	[k]132	65	[l]5	0

[a]Asymptomatic male, on 9/25/85, tested Abbott EIA screening
test negative, Mab assay negative, Abbott CORE (p24) test negative, and
Abbott ENV positive. All tests were negative on serum taken four
months later.
[b]Female contact of a seropositive male.
[c]Young male of Mediterranean descent with Kaposi's sarcoma
not associated with AIDS.
[d]Specimens randomly picked from the Abbott HTLV III EIA
Screening Assay clinical trials, and donor status could reflect
multiple samples from a few individuals.
[e]A longitudinal study.
[f]Serum samples which were Abbott EIA screening assay
positive and Abbott CORE (p24) positive.
[g]Serum sample which was Abbott EIA screening assay positive
and Abbott CORE (p24) negative.

[h]All sera were tested using the Abbott EIA screening assay, of which four were positive. Of those four, two were positive by the ENV and Mab tests, and two were negative by both tests. The remaining 36 sera were all negative by the screening assay and Mab assay, and are assumed to be ENV negative.
[j]A longitudinal, seroconversion study with blood samples drawn every 3 months up to the time of seroconversion. All samples were diluted 1:3 prior to assay due to small sera volumes.
[k]Includes 27 seroconversion points.
[l]All five sera were seroconversion points, three of which were Mab borderline and may have been positive using undiluted sera; the remaining two sera were discrepant.

Table 3 presents an analysis of the sensitivity and specificity of the 5-21-3 Monoclonal Assay based on the data presented in Table 2. A modified calculation of sensitivity and specificity also is presented, which accounts for assay variations, dilutions of clinical specimens, and false positives presented by the ENV Polyclonal Assay. Based on these data, the clinical utility of the Monoclonal Assay was firmly established, in that it detected greater than 99% of all HIV I gp41 seropositives detected by the Polyclonal Assay, had a false negative rate of less than 1%, and was less susceptible to false positives.

Table 3. Sensitivity and Specificity of the Monoclonal Competitive Assay Compared to the ENV Polyclonal Competitive Assay.

ENV Assay	Monoclonal Assay			
	Pos		Neg	
Pos	(432)	437	(6)	*2
Neg	+ (3)	1	(189)	190
Sensitivity	(98.6)			99.5
Specificity	(98.4)			99.5

Original data presented in parentheses; modified data presented to the right of original data.

*1 ENV false positive (Table 2, footnote a) is placed as ENV neg/Mab neg; 3 Mab borderline false negatives (Table 2, footnote l) are placed as ENV pos/Mab pos: the remaining 2 ENV pos/Mab neg samples are two discrepant seroconversion points.

+2 ENV false negative samples (Table 2,
footnote f) are placed as ENV pos/Mab pos; the
remaining 1 ENV neg/Mab pos sample may represent a
Monoclonal assay false positive (Table 2, footnote
g).

The Polyclonal Assay has been evaluated by several
laboratories and has been found to be equivalent to or superior over
current blotting procedures or other EIA assays for detection of HIV I
seropositive individuals (Dienhardt et al., Lancet (1987) Jan. 3:40;
Denis et al., Lancet (1987) Feb. 7:324; Allain et al., Lancet (1986)
Nov. 29:1233- 1236; Habermehl et al., supra; and Dawson et al.,
supra). Therefore based on the high degree of sensitivity of the
Monoclonal Assay, compared to the Polyclonal Assay, it was inferred
that the Monoclonal Assay also was equivalent to or superior over most
other blotting or EIA assays.

Example 7
Mapping and Characterization of the gp41 Epitope Recognized by
Monoclonal Antibody 5-21-3

A. Synthesis of Synthetic Peptides
Fig. 4 illustrates the location of the synthetic peptides
which were made in order to map the epitope recognized by monoclonal
antibody 5-21-3. The synthesis of each peptide is outlined below:
1) Synthesis of the peptide corresponding to gp41 amino acids
121-154.

NH_2-Asp-Arg-Glu-Ile-Asn-Asn-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu
-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-COOH

This polypeptide was assembled on a resin support by stepwise
solid phase synthesis (starting with the carboxy-terminal residue). A

procedure was employed similar to the one described by Barary and Merrifield (E. Gross and J. Meinehofer, Eds., The Peptides, vol. 2 [Academic Press, New York, 1980], p. 1284). A Boc-L-Arg(Tos)-OCH$_2$-Pam resin was transferred to a reaction vessel of an Applied Biosystems Synthesizer, Model 430A. Protected amino acids were coupled in a stepwise manner to the resin support by preformed symmetric anhydride chemistry, except, in the cases of arginine, asparagine, and glutamine addition, where the DCC/HOBT protocol described by Konig and Geiger (Chem. Ber. (1970) 103:788-798) was employed. All amino-terminal residues were protected by t-butyloxy carboxyl (t/BOC)-linkage, and side chains of various amino acid residues were protected by the following groups: Arg, Tos; Lys, 2-ClZ; Glu, OBZl; Thr, Bzl; Tyr, 2-BrZ.

The fully protected peptide-resin (0.5-1.0g) was allowed to swell in methylene chloride (CH$_2$Cl$_2$) for 5 minutes. The N-Boc protecting groups were removed using 60% trifluoroacetic acid (TFA/CH$_2$Cl$_2$) deprotection, CH$_2$Cl$_2$ washes, 10% N,N-diisopropylethylamine (DIEA/CH$_2$Cl$_2$) neutralization, and final CH$_2$Cl$_2$ washes. The resin was dried in vacuo. The peptide-resin thus obtained was treated with 9 ml of anhydrous hydrofluoric acid (HF), to which 1 ml of p-cresol had been added, for 60 minutes at 0°C. The HF was distilled off in vacuo at 0°C. The cleaved, free peptide and resin were washed 3 times with 15 ml aliquots of diethyl ether. The peptide was then extracted three times with 15 ml of 40% aqueous acetic acid. The aqueous extracts were combined, washed three times with 10 ml aliquots of diethyl ether, and the aqueous layer was lyophilized to provide the crude peptide for purification.

The polypeptide was purified by reversed-phase HPLC on C$_4$ columns, employing gradients of 0.1% TFA/water (A) and 100% acetonitrile (B) as the solvent systems at a flow rate of 1 ml/min for the analytical column (Vydac-214-TP54, Vydac Separation Group, Hesperia, California) or 3 ml/min for the semi-preparative one (Vydac-214-TP510).

The gradient used was:

28% B   1min   28%B   20min   47%B   1min   28%B

The polypeptide elution from the HPLC column was monitored at 225 nm
and 280 nm. The composition of the polypeptide was confirmed by
hydrolysis in 6 N hydrochloric acid (HCl)/0.3% phenol at 150°C for 2 hr
in vacuo, and subsequently analyzed on a Beckman 6300 amino acid
analyzer with a SICA 7000 A integration.
2) Synthesis of the peptide corresponding to gp41 amino acids 126-162.

NH_2-Tyr-Asn-Tyr-Thr-Ser-Leu-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln
-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-Trp-Ala-Asn
-Leu-Trp-Asn-Trp-Leu-COOH

        This sequence of the peptide was assembled on the solid
support by essentially the same procedure described above. The amino
acid tryptophan was protected by the formyl (CHO) group. Double
coupling protocols were used for amino acids underlined in the sequence
shown above. The desired peptide was deprotected and cleaved off as
described for the first peptide except that the peptide-resin was
treated with a mixture of 8.5 ml HF, 1.0 ml of p-cresol and 0.5 ml
ethanedithiol. The cleaved peptide was extracted using 15% and 40%
aqueous acetic acid. The crude peptide so obtained was analyzed and
purified on a C_4 reversed-phase column as described above except the
gradient used was:

35%B   1 min   35%B   20 min   70%B   2 min   35%B

                        concave   linear
                        curve

3) Synthesis of peptide corresponding to gp41 amino acids 131-154.

NH$_2$-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-
Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-COOH

      This peptide was assembled, cleaved, and purified as described
for the first peptide, except that the following gradient was used for
analysis and purification:

15%B 1 min 15%B   20 min 40%B 2 min 40%B   2 min 15%B
                      linear   linear

4) Synthesis of peptide corresponding to gp41 amino acids 67-154.

NH$_2$-Ala-Arg-Ile-Leu-Ala-Val-Glu-Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-
Gly-Ile- Trp-Gly-Cys-Ser-Gly-Lys-Leu-Ile-Cys-Thr-Thr-Ala-Val-Pro-Trp-
Asn-Ala-Ser-Trp- Ser-Asn-Lys-Ser-Leu-Glu-Gln-Ile-Trp-Asn-Asn-Met-Thr-
Trp-Met-Glu-Trp-Asp-Arg-Glu-Ile-Asn-Asn-Tyr-Thr-Ser-Leu-Ile-His-Ser-
Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-Gln-Glu-Leu-Leu-
Glu-Leu-Asn-Lys-COOH

      This peptide was assembled on the solid support as described
in (1) above with the following differences. A single coupling
protocol was used for assembling amino acids from 154 to 143 except for
asparagine and glutamine which were coupled twice. Double coupling
protocols were used for all subsequent amino acids from 142 to 67.
During this synthesis, 0.45 g and 0.60 g of the resin, respectively,
were taken out after the addition of amino acids aspartic acid at
position 121 and serine at position 104. The integrity of the
assembled peptide was confirmed by solid phase sequencing (11 to 18
cycles) during synthesis. Specifically, the peptide-resin was
sequenced (i) after adding amino acid aspartic acid at position 121
(121-154), (ii) after adding amino acid leucine at 82 (82-154), and
(iii) at the end of the synthesis (67- 154). In all cases, the
overwhelming major sequence observed was the desired one, with minor
amounts of deletion sequences.

The desired peptide was deprotected and cleaved off the resin using the "low high HF" procedure as described by Tam et al., J. Am. Chem. Soc. (1983) 105:6442-6455. After evaporation of HF, the reaction mixture was washed with ether followed by extraction of the free peptide with 40% aqueous acetic acid. The resultant acetic acid extract, which was turbid with precipitates, was centrifuged to obtain an insoluble pellet. The supernatant was lyophilized to obtain a white crude peptide. The insoluble pellet was dried in a lyophilizer overnight to also yield crude peptide.

The crude peptide (67-154) obtained above was dissolved in an 8 M urea, 50 mM Tris-HCl, pH 8.2, buffer containing 50 mM dithiothreitol (DTT). This solution was incubated at 42°C for 1 hr, and then dialyzed in a spectrapor membrane (cut-off range 6000-8000) against the following buffers:

(i) 4 M urea, 50 mM Tris-HCl, pH 8.2, 50 mM DTT for 16-18 hr;

(ii) 3 M urea, 50 mM Tris-HCl, pH 8.2, 50 mM DTT for 48 hr;

(iii) 50 mM Tris-HCl, pH 8.1, for 6-10 hr with 4 exchanges of fresh buffer.

The stock solution of the peptide thus obtained was reduced with 50 mM DTT at 40°C for 90 minutes. The solution was brought to room temperature, and then dialyzed in a spectrapor membrane (cutoff 6500- 8000) against a 0.1 M ammonium acetate buffer, pH 8.1, for 48 hr. The buffer was changed twice. After a total of 72 hr of dialysis, the peptide solution was diluted 3-fold with 0.1 M ammonium acetate buffer, pH 8.1, and allowed to stand in air for 48 hr. A UV spectrum of this peptide solution in water showed a maxima at 276 nm with a shoulder at 289 nm. The peptide was further purified on a reversed-phase C_4 column, and then analyzed as described above, using the following gradient:

30%B   1 min   30%B   20 min   65%B   1 min   30%B

5) Synthesis of peptide corresponding to gp41 amino acids 58-130.

NH$_2$-Thr-Val-Trp-Gly-Ile-Lys-Glu-Leu-Gln-Ala-Arg-Ile-Leu-Ala-Val-Glu-
Arg-Tyr-Leu-Lys-Asp-Gln-Gln-Leu-Leu-Gly-Ile-Trp-Gly-Cys-Ser-Gly-Lys-Leu-
Ile-Cys-Thr-Thr-Ala-Val-Pro-Trp-Asn-Ala-Ser-Trp-Ser-Asn-Lys-Ser-Leu-Glu-
Gln-Ile-Trp-Asn-Asn-Met-Thr-Trp-Met-Glu-Trp-Asp-Arg-Glu-Ile-Asn-Asn-Tyr-
Thr-Ser-Leu-COOH

6) Synthesis of peptide corresponding to gp41 amino acids 131-175.

NH$_2$-Ile-His-Ser-Leu-Ile-Glu-Glu-Ser-Gln-Asn-Gln-Gln-Glu-Lys-Asn-Glu-
Gln-Glu-Leu-Leu-Glu-Leu-Asp-Lys-Trp-Ala-Asn-Leu-Trp-Asn-Trp-Leu-Asn-Ile-
Thr-Asn-Trp- Leu-Trp-Tyr-Ile-Lys-Leu-Phe-Ile-COOH

    Synthesis and purification of these peptides proceeded using the methods outlined above.

B. Epitope Mapping of Antibody 5-21-3 by Immuno-Dot Blotting Using Synthetic Peptides

    The western Blot procedure described in Example 1 was employed for development of immuno-dot blots using synthetic peptides, except that synthetic peptides were fixed to nitrocellulose by direct application using Drummond lambda pipettes, and air dried prior to blocking with 20% fetal calf serum in PBS (pH 7.4). Monoclonal IgG 5-21-3, and a second anti-HIV I gp41 monoclonal antibody (IgG) 10-15-63 (which recognizes an epitope distinct and separate from the epitope recognized by 5-21-3), were purified from mouse ascites using Protein A-Sepharose, and employed at the concentrations indicated.

    Immuno-dot blot results are illustrated in Figure 5 where synthetic peptides were spotted onto nitrocellulose as follows:(A) 10 ug P58-130, (B) 10 ug P67-154,(C) 6 ug P121-154 and (D) 9 ug of P126-162. The strips were reacted with 25 ug/ml monoclonal antibody 5-21-3, 25 ug/ml monoclonal antibody 10-15-63, 25 ug/ml normal mouse

IgG, 2% mouse (M) anti-rp41 serum, 2% normal mouse serum (NMS), 2% Rabbit (R) anti-P58-130 serum, or 2% normal rabbit serum (NRS). Dot blot results (Fig. 5) clearly demonstrated that antibody 5-21-3 did not react with P58-130. In sharp contrast, addition of amino acids 131-154 to the carboxy end to form P67-154 (which lacks 10 amino acids on the amino end) produced a very strong reaction with antibody 5-21-3. Strong reactivities against both P58-130 and P67-154 were detected using rabbit anti-P58-130 serum and mouse anti-rp41 serum, demonstrating that the peptides were present on the nitrocellulose. Little or no reactivity against either peptide could be demonstrated using monoclonal antibody 10-15-63, normal mouse IgG, normal mouse serum, or normal rabbit serum. Under conditions employing mild washing procedures, additional 5-21-3 reactivity was observed against the smaller P126-162, and a faint, diffuse reaction against P121-154. Because smaller peptides may not bind tightly to nitrocellulose, reactivity of 5-21-3 by dot blot against smaller peptides, for example P121-154, P131-154, and P131-175 (not shown) was difficult to demonstrate. However, it is clear that addition of amino acids 131-154 to a larger peptide(67-154) provided an epitope recognized by 5-21-3, and that no reactivity was demonstrated against a similar peptide (P58-130) which lacked amino acids 131-154.

C. Radiolabeling of Synthetic Peptides

Synthetic peptides were radiolabeled with ^{125}I (Amersham) by the chloramine T method (W.M. Hunter, "Radioimmunoassay" in D.M. Weir, Ed., Handbook of Experimental Immunology, [Blackwell Scientific Publications, 1973]). Generally, 2-6 ul of 100 mCi/ml ^{125}I were added to 10-50 ug of peptide in PBS, pH 7.4. Approximately 10 ug of freshly prepared (1 mg/ml in PBS) chloramine T were added to the mixture maintained at 4°C. After 5 min., $[^{125}I]$peptide was separated from free ^{125}I by passage of the reaction mixture through a 3 ml (bed volume) column of Biogel P-6 or P-2, as required. Fractions (200 ul each) containing $[^{125}I]$peptide were pooled and diluted into PBS

containing 20% fetal calf serum. Rough estimations of ^{125}I cpm bound
to peptide were made by acid precipitation. An aliquot of labeled
peptide was diluted into 1 ml of ice cold 50% trichloroacetic acid
(TCA) overlaying a 0.45 um filter (made of mixed cellulose esters) set
on a scintered glass filter connected to a vacuum apparatus.
Precipitated protein was fixed onto the filter by application of
vacuum, and filters were washed with ice-cold 50% TCA. Background cpm
adhering non-specifically to the filter were estimated by repeating the
procedure using a non-precipitating buffer. Amounts of peptide, ^{125}I,
chloramine T, and composition of column eluting buffer were subject to
change depending on conditions required to maintain solubility of each
peptide.

D. Epitope Mapping of 5-21-3 by Immunoprecipitation of Radiolabeled Synthetic Peptides

Synthetic peptides radiolabeled with ^{125}I were diluted into PBS
(pH 7.4) containing 0.1% Triton X-100 (or other detergents where
indicated) and 1.0% BSA, to yield approximately $2.5-5 \times 10^5$
acid-precipitable cpm per 0.1 ml. Purified monoclonal IgG 5-21-3 and
10-15-63 were employed in the assay. Monoclonal antibodies were
diluted into PBS containing 0.1% Triton X-100 (or other detergents
where indicated), 1.0% BSA, and 2% normal mouse serum as a carrier
immunoglobulin. Immune rabbit serum (anti-P58-130) was diluted
appropriately into the same buffer except that normal rabbit serum (2%)
was substituted for normal mouse serum as the carrier immunoglobulin.
A reaction mixture comprising 0.1 ml [^{125}I] peptide (5×10^5
cpm/0.1 ml) and 0.1 ml diluted antibody was incubated 1 hr at 40°C,
followed by addition of 0.1 ml of a precipitating antibody, either goat
anti-mouse IgG, or goat anti-rabbit IgG, corresponding to the primary
antibody. The reaction mixture was incubated 1 hr at 40°C and then
overnight at 4°C. The precipitate was collected by centrifugation
(2,000 x g, 5 min., 20°C), washed once in PBS containing 0.1% Triton
X-100 and 1.0%

BSA, collected by centrifugation, and counted in an LKB 128 gamma counter. Precipitation assays employing Protein A-Sepharose as the precipitating reagent were performed in a similar manner, except that normal sera were omitted from the reaction mixtures.

In agreement with the dot blots discussed above, antibody 5-21-3 was unable to bind P58-130 (see Table 4), indicating that the amino acids which form the epitope are not present in this peptide. In sharp contrast, and in agreement with the dot blot data, addition of amino acids 131-154 to the carboxy end of P58-130 to form P67-154, produced an epitope which was readily bound by the 5-21-3 antibody. Antibody 5-21-3 was unable to bind P131-154 which contains only those amino acids which form the epitope. However, the addition of flanking sequences, 10 amino acids to the amino end of 131-154 to form P121-154, as well as 5 and 8 amino acids to the amino and carboxy ends, respectively, to form P126-162, provided an epitope which was recognized by 5-21-3. Little or no binding activity by 5-21-3 against P131-175 was detected. These data indicated that the correct linear amino acid sequence (P131-154) was not sufficient for recognition and binding of 5-21-3, but rather, additional sequence either 5' (amino end) of amino acid 131 or 3' (carboxy end) of amino acid 154, were required to put the linear sequence in the proper antigenic conformation for recognition by 5-21-3. For all peptides, specific binding using monoclonal 10-15-63 could not be detected. In addition, rabbit anti-P58-130 was a strong precipitator of P58-130, P67-154, and P121-154, indicating the presence of precipitable peptide.

Table 5 illustrates the effect of antibody concentration on binding activity of 5-21-3. Clearly, while as little as 1 ug/ml of 5-21-3 bound significant amounts of P67-154, at least 100 ug/ml of the antibody was required to demonstrate even low level reactivity against the smaller peptides P121-154 and P126-162, and reactivity against P131-154 could not be demonstrated. These data indicate that 5-21-3 may have a much greater affinity for its epitope when the epitope is presented as part of a larger peptide, as opposed to identical sequence presented as a much shorter peptide.

Table 4. Precipitation of [^{125}I] Synthetic Peptides by Monoclonal Antibody 5-21-3.[a]

SPECIFIC PRECIPITATION (CPM)[b]

[^{125}I]PEPTIDE	5-21-3	10-15-63	R⋅P58-130
P58-130[c]	0 (<1)	0 (<1)	286,722 (32)
P67-154[d]	479,207 (16)	9713 (1.3)	479,343 (4.4)
P131-154[d]	970 (1.1)	0 (<1)	0 (<1)
P121-154[d]	40,717 (31)	371 (1.3)	310,419 (24)
P126-162[d]	11,540 (3.4)	0 (<1)	0 (<1)
P131-175[d]	317 (1.6)	188 (1.4)	0 (<1)

[a]Monoclonal antibodies 5-21-3 and 10-15-63 (anti-gp41 recognizing an epitope distinct and separate from that recognized by 5-21-3) were purified from mouse ascites and used at 10 ug/0.2 ml reaction mixture. Rabbit (R) anti-P58-130 serum was used at 20 ul/0.2 ml reaction mixture.

[b]Specific precipitation was calculated as (specific cpm - non-specific cpm), where non-specific cpm was determined using normal mouse IgG (10 ug/0.2 ml) or normal rabbit serum (20 ul/0.2 ml). Numbers in parentheses represent the x-fold increase of specific precipitation (using 5-21-3, 10-15-63, or R anti-P58-130) over non-specific precipitation (using normal mouse IgG or normal rabbit serum). Between 250,000 and 500,000 trichloroacetic acid precipitable cpm of iodinated peptides were routinely employed per reaction mixture.

[c]Precipitation of P58-130 was performed using PBS buffer (pH 7.4) containing 0.1% Triton X-100, 1.0% sodium deoxycholate, and 0.1% SDS. Immune complexes were collected using Protein A- Sepharose. The detergent-rich buffer was chosen in order to retain solubility of P58-130.

[d]Precipitation of these peptides was performed using PBS buffer (pH 7.4) containing 0.1% Triton X-100, 1.0% bovine serum albumin, and 1.0% (final concentration) normal mouse or rabbit serum as carrier protein. Immune complexes were precipitated using a second antibody, either goat anti-mouse IgG, or goat anti-rabbit IgG.

Table 5. Effect of 5-21-3 Antibody Concentration on Binding of Synthetic Peptides.[a]

SPECIFIC PRECIPITATION (CPM)[b]

[125I]PEPTIDE	5-21-3 (ug/ml)			10-15-63 (ug/ml)		
	1.0	10.0	100.0	1.0	10.0	100.0
P67-154	165,781 (3.6)	441,887 (8.5	659,045 (8.0)	0 (0)	0 (0)	0 (0)
P121-154	0 (0)	1,113 (2.3)	18,984 (13)	0 (0)	0 (0)	0 (0)
P126-162	0 (0)	295 (1.1)	4,924 (1.6)	0 (0)	0 (0)	0 (0)
P131-154	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	112 (1.1)

[a]Precipitation of these peptides was performed using PBS buffer (pH 7.4) containing 0.1% Triton X-100, 1.0% bovine serum albumin, and 1.0% (final concentration) normal mouse serum as carrier protein. Immune complexes were precipitated using goat anti-mouse IgG.
[b]Monoclonal antibodies 5-21-3 and 10-15-63 (anti-gp41) were purified from mouse ascites. The values (ug/ml) given for each antibody represent the final concentration of the antibody in the reaction mixture. Specific precipitation was calculated as (specific cpm - non-specific cpm), where non-specific cpm was determined using normal mouse IgG. Numbers in parentheses represent the x-fold increase of specific precipitation using 5-21-3 or 10-15-63, over non-specific precipitation using normal mouse IgG.

E. Epitope Mapping Using Synthetic Peptides to Inhibit Binding of 5-21-3 to rp41

Additional evidence for the conformational nature of the 5-21-3 epitope was obtained using binding inhibition studies, in which synthetic peptides were ranked for their ability to inhibit binding of 5-21-3 to rp41. First, the Fab dimer of an anti-gp41 monoclonal antibody was prepared by conventional methods (Goding et al., supra). Then 100 ul of a 1 mg/ml solution of the Fab dimer in bicarbonate

buffer (pH 9.6) was coated onto Immulon II microtiter plates for 1 hr at 37°C. After washing with PBS containing 0.05% Tween 20, the plates were overcoated with 200 ul of 1.0% ovalbumin in bicarbonate buffer (pH 9.6) for 1 hr at 30°C. After washing, 100 ul of rp41 enriched E. coli lysate (as described in Example 3), diluted appropriately in PBS containing 0.05% Tween 20, were allowed to bind to the Fab dimer for 2 hr at 30°C. The plates were washed and stored at 4°C.

Intact monoclonal antibody 5-21-3 IgG (purified from tissue culture fluid supporting growth of the cloned cell line 5-21-3) was titrated against the bound rp41 on the plate, and an antibody concentration was selected which yielded an A_{492} value of 2.0-3.0. Usually, 0.4 pmol (0.06 ug) of 5-21-3 IgG per microtiter well was employed in these assays. Stock solutions of 5-21-3 IgG were prepared in PBS containing 0.1% Triton X-100 and 0.1% SDS. Dilutions of synthetic peptides were made in the same buffer (SDS was included in these experiments to keep peptides soluble at high concentrations).

The binding inhibition assay was performed by combining 0.15 ml of 1.2 ug/ml 5-21-3 antibody stock solution with 0.15 ml of peptide at a selected peptide concentration, and incubating the reaction mixture 1 hr at room temp. Then 0.1 ml aliquots of each reaction mixture were added to each of two wells in the microtiter tray. After a 1 hr incubation at room temp., the plates were washed, and HRPO conjugated Goat anti-mouse IgG Fc was added for 1 hr at room temp. The plates were washed, and addition of OPD for color development identified the relative amounts of 5-21-3 bound to rp41 in the microtiter wells. The reaction was stopped by addition of 100 ul of 1N H_2SO_4, and the plates were read on a Dynatech II microtiter plate reader.

Consistent with previous dot blot and immunoprecipitation results, Fig. 6 demonstrates the lack of reactivity of P58-130 with 5-21-3. There was no detectable inhibition of 5-21-3 binding to rp41 when P58-130 was employed as the inhibitor. In sharp contrast, P67-154, which contains the additional carboxy-terminal amino acids

131-154, was a potent inhibitor of binding activity. In addition, P121-154 also was a potent inhibitor of binding, although considerably more peptide was required to elicit 50% inhibition, compared to P67-154.

Table 6 ranks the synthetic peptides for their ability to block binding activity of antibody 5-21-3. Consistent with the previous data, P67-154 was the most potent inhibitor, but was considerably less effective than native antigen (rp41). In addition, although P131-154 contains the same linear sequence of amino acids as the carboxy end of P67-154, inhibition of 5-21-3 with this peptide could not be demonstrated even up to a 50,000-fold molar excess of peptide. Furthermore, the addition of a flanking sequence, consisting of 10 amino acids 5' (amino end) to amino acid 130 to produce P121-154, restored binding inhibition activity by at least 20-65 fold, although P121-154 was still 20-fold less active than P67-154. The binding inhibition activity of P126-162, which contains 5 and 8 additional amino acids 5' (amino end) and 3' (carboxy end) of amino acids 130 and 154, respectively, was roughly equivalent to that of P121-154. Importantly, P131-175, which contains no additional amino acids 5' of amino acid 131, was a definite inhibitor of 5-21-3 binding activity, although only at high concentrations. Flanking sequence 3' (carboxy end) of amino acid 154 in P131-175, apparently contributes to the antigenic conformation of the 131-154 epitope, as does sequence 5' (amino end) of amino acid 131 (for example, P121-154). More importantly, amino acids on the 5' (amino) side of amino acid 131 are not absolutely required in order to identify the location of the amino acids which will ultimately form the native epitope recognized by the monoclonal antibody when sufficient flanking sequences are present.

Table 6. Relative Concentrations of Synthetic Peptides Required to Inhibit Binding of Antibody 5-21-3 to rp41.

Inhibitor (Peptide)	Molar Ratio[a]
rp41	9.5
P67-154	72.7
P131-154	>50,000
P121-154	1,494
P126-162	1,585
P131-175	18,815
P194-220[b]	>50,000

[a]Ratios are expressed as pmoles peptide/pmoles 5-21-3, at 50% inhibition of 5-21-3 binding; each number represents the mean of two experiments.

[b]This peptide, used as a control, was synthesized from a region outside the epitope of 5-21-3.

Results of the immuno-dot blot, immunoprecipitation, and binding inhibition assays, all indicate that the amino acids which form the epitope recognized by monoclonal antibody 5-21-3 lie 3' of amino acid 130 (the HindIII site), and 5' of amino acid 154. Proper conformation of the amino acids comprising this region is essential to form the epitope recognized by antibody 5-21-3. The conformation-dependent nature of the epitope is evidenced by the marked influence of flanking sequences (5' of amino acid 131 or 3' of amino acid 154) on binding of antibody 5-21-3 to peptides containing sequence 131-154. Further, linear sequences comprising the region of interest, amino acids 131-154, do not by themselves form the epitope recognized by the 5-21-3 antibody. Thus, the linear sequence is not antigenic, and by inference, does not form the antigenic determinant found in the native protein which was used as the immunogen for production of B cells secreting 5-21-3 antibody. By analogy, human antibodies elicited by native gp41, which compete with antibody 5-21-3, also must recognize the native antigenic determinant which is conformation dependent, and is identical to or indistinguishable from the epitope recognized by

antibody 5-21-3. Proper evaluation of human sera which recognize the
5-21-3 epitope (as determined by their ability to compete with 5-21-3)
by methods other than competition with 5-21-3, must take into account
that short linear peptides or cloned gene fragments which do not
contain sufficient sequence cannot present the native epitope.
Recombinant proteins which utilize and begin at the Hind III site in
gp41 will not form the complete native epitope, nor can the fully
antigenic native epitope be detected using such recombinant proteins.
Similarly, synthetic peptides claiming linear or conformational
epitopes which comprise the sequence between amino acids 131-154
without sufficient flanking sequences cannot represent the fully
antigenic, native epitope. Apparently, native or near full-length
recombinant gp41 may be required to form the native epitope which is
recognized by 5-21-3 and which is clinically useful as a serological
target.

Changes and modifications may be conceived and practiced
without departing from the spirit and scope of the invention and it is
understood that such equivalent embodiments are intended to be included
herein.